Displacement of itraconazole from cyclodextrin complexes in biorelevant media: in vitro evaluation of supersaturation and precipitation behavior

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Abstract

Intestinal fluids contain several constituents with affinity for cyclodextrins and therefore have the potential of displacing drugs from the cyclodextrin cavity by competition. In this study, the solubilizing capacity of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) for itraconazole was studied in presence of selected bile salts and phosphatidylcholine. Despite the fact that these competing agents significantly lowered the solubility of itraconazole in presence of cyclodextrins, the addition of concentrated solutions of these bile constituents to a solution containing itraconazole solubilized by HP-β-CD did not result in precipitation, even at bile salt and phospholipid concentrations where itraconazole precipitation would be anticipated based on solubility studies. This phenomenon was further investigated in more dynamic conditions via in vitro transfer studies, mimicking the gastrointestinal transfer of HP-β-CD solutions saturated with itraconazole. Intestinal supersaturation upon transfer was observed for all conditions tested and a concentration dependent impact of bile salts and phospholipids on the precipitation behavior of itraconazole was demonstrated: high concentrations of bile salts and phospholipids generated the highest degrees of supersaturation shortly after the transfer step but also resulted in stronger itraconazole precipitation at later time points. These findings demonstrate the possible impact of the variable intestinal fluid composition on the behavior of cyclodextrin containing formulations.

Keywords (6): itraconazole, cyclodextrin, biorelevant media, gastrointestinal transfer, supersaturation, precipitation

1 Introduction

Cyclodextrins (CDs) continue to attract a lot of attention from formulation scientists because of their excellent solubilizing capacity; they have been used in a multitude of marketed pharmaceutical products including drugs intended for oral, intravenous, intramuscular or topical administration (Loftsson and Brewster, 2010). The central cavity of these cyclic oligosaccharides can be considered a hydrophobic pocket, which plays a crucial role in improving the solubility of lipophilic drugs. Through formation of inclusion complexes with these lipophilic compounds, CDs may significantly enhance the bioavailability of drugs exhibiting poor dissolution characteristics or a limited aqueous solubility. Moreover, recent observations of spontaneous CD self-assembly suggest that these aggregates can take the form of micelle-like structures contributing to the solubility of guest compounds through non-inclusion complex formation (He et al., 2008; Loftsson et al., 2004; Ryzhakov et al., 2016).

Although several successful CD containing formulations have been marketed for oral administration, the gastrointestinal behavior of drugs in presence of CDs is complex and sometimes difficult to predict. Solubilization of poorly soluble drugs does not necessarily guarantee an improved intestinal absorption as an increase in solubility often goes hand in hand with a decrease in the free, bioaccessible fraction of the compound (Katneni et al., 2006; Miller et al., 2011; Yano et al., 2010). Beig et al. demonstrated that this solubility-permeability interplay is indeed valid for lipophilic compounds in presence of CDs (Beig et al., 2013). Since the intestinal absorption of CDs (and CD complexes) is very low to non-existent, the drug has to be released from the hydrophobic cavity to obtain a satisfactory uptake. Therefore, very high binding constants or supraoptimal CD concentrations may impede intestinal absorption.

Stella et al. described the mechanisms by which drugs can be released from CD complexes and mentioned dilution and competitive displacement of drugs from the hydrophobic cavity as the main factors driving dissociation between drug and CD (Stella et al.,

1999). Although not quite as significant as the dilution factor upon intravenous administration, a formulation may undergo considerable dilution upon oral ingestion. Factors influencing the extent of dilution of CDs in a formulation include the volume of the residual gastric fluids and the possible concomitant intake of water with the formulation. Moreover, Hens et al. recently demonstrated that in fasted state conditions, the dilution factor upon gastrointestinal transfer is rather limited and lies close to 1:1 (Hens et al., 2014).

Intestinal fluids contain several agents which have the potential of displacing drugs from the hydrophobic cavity of CDs, such as bile salts and phospholipids. Furune et al. demonstrated that α-CDs extract phosphatidylcholine from the mixed micelles present in the intraluminal environment, hereby diminishing the solubilizing capacity of the intestinal fluids (Furune et al., 2014). In addition to phospholipids, bile salts also exhibit affinity towards CDs. A series of detailed studies describes the interaction between the most prevalent bile salts in humans and natural or substituted CDs (Holm et al., 2011, 2007; Schönbeck et al., 2010). A recently performed study by Olesen et al. rightfully underscores the fact that the intraluminal presence of bile salts in the small intestine affects the concentration of CD that is theoretically required to solubilize a drug of interest (Olesen et al., 2015). Although this work relies on a number of mathematical and physical assumptions, it is a useful reminder for formulation scientists to consider the complexity of the intraluminal environment.

The highly variable nature of the intestinal fluids was further emphasized by Riethorst et al. who performed an in-depth characterization of duodenal aspirates from twenty healthy volunteers (Riethorst et al., 2016). The strongly scattered individual concentrations of different bile salts, cholesterol, phospholipids and lipid degradation products measured in the intestinal media advocate the implementation of this variability into the experimental design of in vitro experiments. Despite the fact that these competing agents may severely influence the thermodynamic solubility of drugs in presence of CDs, drug displacement does not necessarily

result in immediate or irreversible precipitation of the drug. For example, Miyake et al. demonstrated superior absorption of cyclosporine A from a cyclodextrin containing formulation in rats with continuous bile flow as compared to bile duct-cannulated rats (Miyake et al., 1999). Moreover, the identification of CDs as suitable precipitation inhibitors, renders this hypothesis of drug supersaturation upon displacement from CDs all the more likely (M. E. Brewster et al., 2008).

The aim of this study was to evaluate the solubilizing capacity of HP-β-CD for itraconazole in presence of varying biorelevant concentrations of bile salts and phospholipids. The antifungal agent itraconazole is marketed as a solution containing 40% of HP-β-CD to solubilize 10 mg/ml of itraconazole. Itraconazole has a very poor aqueous solubility which increases significantly upon addition of HP-β-CD (Marcus E. Brewster et al., 2008). In this investigation, the capacity of bile salts and phospholipids to displace itraconazole from the hydrophobic cavity of the CDs was assessed. In addition, the effect of displacement by competing agents on itraconazole supersaturation/precipitation behavior was investigated in vitro.

2 Materials and methods

2.1 Chemicals

Itraconazole and 2-hydroxypropyl-β-CD (HP-β-CD) were kindly provided by Johnson & Johnson (Beerse, Belgium). The degree of substitution of HP-β-CD was 0.63. Sigma Aldrich (St. Louis, MO) supplied sodium taurocholate (TC), sodium glycocholate (GC), sodium glycodeoxycholate (GDC) and sodium glycochenodeoxycholate (GCDC). Sodium acetate trihydrate and methanol were purchased from VWR International (Leuven, Belgium). Simulated intestinal fluids (SIF) powder was obtained from Biorelevant (Croydon, UK). Water was purified using a Maxima system (Elga Ltd., High Wycombe Bucks, UK).

2.2 Media

Fasted (FaSSIF) and fed (FeSSIF) state simulated intestinal fluids and fasted state simulated gastric fluids (SGF) were made according to the manufacturer's preparation protocol (Biorelevant®, Croydon, UK). FaSSIF was prepared by dissolving SIF powder (2.24 mg/mL) in a phosphate buffer (28.4 mM, pH 6.5) and a pH modified version of FeSSIF was prepared by dissolving SIF powder (11.2 mg/mL) in the same phosphate buffer (pH 6.5) instead of the commonly used acetate buffer (pH 5). This was done to exclude the pH as a possible confounding factor. SGF was made by dissolving SIF powder (0.06 mg/mL) in an HCl/NaCl solution (pH 1.6). SIF powder contains the bile salt taurocholate and the phospholipid phosphatidylcholine at a ratio of 4:1. A concentration of 1 mg SIF powder per ml of medium is equivalent with 1.34 mM taurocholate and 0.33 mM phosphatidylcholine. Consequently, FaSSIF contains 3 mM of taurocholate and 0.75 mM of phosphatidylcholine and FeSSIF contains 15 mM of taurocholate and 3.75 mM of phosphatidylcholine.

To correct for the 1:1 dilution effect during the gastrointestinal transfer studies, a series of double concentrated intestinal media were prepared by dissolving 5, 15 and 20 mg of SIF

powder per ml of a double concentrated phosphate buffer (56.8 mM, pH 7.5). A control condition without SIF powder was also included. The pH was set at 7.5 to end up with media at a pH of 6.5 containing 0, 2.5, 7.5 and 10 mg of SIF powder per ml after addition of equal volumes of SGF.

2.3 Solubility measurements

The apparent solubility of itraconazole was determined in several biorelevant media, including simulated gastric and intestinal fluids in presence or absence of varying concentrations of HP-β-CD, bile salts and phospholipids as well as in blank transfer media. The latter are the media resulting from the 1:1 dilution of SGF containing varying concentrations of HP-β-CD in the double concentrated phosphate buffer (pH 7.5) containing varying concentrations of bile salts and phosphatidylcholine.

All solubility experiments were performed in triplicate. Approximately 3 mg of itraconazole was added to microcentrifuge tubes containing 0.5 ml of medium and placed in a prewarmed shaking incubator [37°C at 175 rpm (KS 4000 i control incubator from Ika (Staufen, Germany))] for 24 h. The solid phase was separated from the dissolved part using centrifugation (15 min, 20.817 g at 37°C). The top layer was carefully removed by aspiration. The supernatant was diluted 1/10 or 1/100 in methanol:water (50:50 v/v) and itraconazole was quantified using an HPLC system with UV detection.

2.4 Displacement studies

The displacement of itraconazole from the CD cavity of HP-β-CD was evaluated by adding concentrated solutions of competing agents to a predefined itraconazole concentration solubilized by a predefined concentration of HP-β-CD. More specifically, a concentrated solution of SIF powder or selected bile salts in phosphate buffer was titrated into a solution of

20 µM or 40 µM, respectively, of itraconazole in 4% HP-β-CD. The concentrated solutions were prepared in such a way that the final concentrations of the media upon titration amounted to 4.1, 5.9, 7.7, 9.5 or 11.2 mg of SIF per ml of buffer or 10 mM of TC, GC, GDC or GCDC per ml of buffer. Experiments were performed in test tubes placed in a water bath maintained at 37°C. Media were stirred using magnetic stirring bars rotating at 400 rpm. Samples (50 µl) were taken from the media at predetermined time points and were centrifuged immediately (10 min, 20.817 g at 37°C). The supernatant was diluted 1/100 in methanol:water (50:50 v/v) and quantified using an HPLC system with UV detection.

2.5 In vitro transfer studies

In vitro transfer studies to mimic the gastrointestinal transfer were performed under various conditions. These experiments are referred to as 'gastrointestinal transfer studies' throughout the manuscript. The gastric compartment consisted out of SGF in absence of CDs or in presence of 2%, 4%, 8% or 12% HP-β-CD saturated up to 75-80% with itraconazole. Incomplete saturation was selected as this significantly decreased the preparation time of the media used in the gastric compartment. The intestinal compartment contained double concentrated phosphate buffer (pH 7.5) in absence or in presence of 5mg or 20 mg SIF powder per ml of phosphate buffer. Both gastric and intestinal fluids were prewarmed in test tubes in a water bath maintained at 37°C. The transfer experiment was initiated by adding 1 ml of the gastric compartment to 1 ml of the intestinal compartment. Upon dilution, the pH of the simulated intestinal fluids dropped from 7.5 to 6.5. The intestinal compartment was stirred using magnetic stirring bars rotating at 400 rpm. Samples (50 µl) were taken from the medium at predetermined time points and immediately centrifuged (10 min, 20.817g at 37°C). The supernatant was diluted 1/100 in methanol:water (50:50 v/v) and quantified using an HPLC system with UV detection.

For the concentrations measured in the intestinal compartment during the transfer studies, degrees of supersaturation (DS) were also calculated. The DS was calculated by dividing the concentration measured at a particular time point, by the equilibrium solubility of the corresponding drug in exactly the same medium.

2.6 Analysis

Itraconazole concentrations were determined by reversed phase HPLC with UV detection. A Hitachi LaChrom Elite HPLC system was used consisting of an L-2130 pump, an L-2200 autosampler and an L-2400 UV detector and EZChrom Elite was used as the software program to integrate the itraconazole peaks (VWR, Leuven, Belgium). The used column was a Waters Nova-Pak® RP-18 (100 x 8 mm, 4 µm) inserted into a radial compression module (Waters, Milford, MA, USA). For all samples, an injection volume of 95 µl was used. The flow rate was set at 1.4 ml/min. The mobile phase consisted of a 25 mM acetate buffer (pH 3.5) and methanol used at a ratio of 82:18. Itraconazole concentrations were monitored by UV absorbance measurement at a wavelength of 265 nm and the retention time was 6.5 min. Calibration curves were linear over the range of 5 µM to 40 nM. The assessment of intraday repeatability, determined at 0.1 and 1 µM resulted in a relative standard deviation (n=5) of 8.3 and 1.1%, respectively; the deviation from the theoretical concentration amounted to 6.7 and 1.8%, respectively.

3 Results and discussion

The capacity of HP-β-CD to solubilize itraconazole in a biorelevant environment was evaluated by performing solubility, displacement and gastrointestinal transfer studies using simulated media reflecting the composition of human gastric and intestinal fluids. While solubility studies are indicative for the concentrations that can be reached in a saturated system in equilibrium, displacement and gastrointestinal transfer studies reflect the dynamic nature of the gastrointestinal tract and concentrations measured in these experiments may deviate significantly from the apparent solubility**.** Moreover, in the solubility determinations, the experiments are initiated by the addition of itraconazole powder to a solution of CDs and/or bile constituents whereas in the displacement and transfer experiments, the solutions initially contain CDs and itraconazole after which they are brought into contact with bile salts and/or phospholipids by titration (displacement studies) or through a transfer step from the stomach compartment to the intestinal compartment (transfer studies).

3.1 Solubility of itraconazole in complex CD containing biorelevant media

In a first approach to evaluate the impact of phospholipids and bile salts on the solubilizing capacity of HP-β-CD for itraconazole, solubility measurements were performed in FaSSIF and FeSSIF in presence of increasing concentrations of HP-β-CD. FaSSIF and FeSSIF are well characterized simulated intestinal fluids representing the fasted and fed state, respectively, and they are widely used for dissolution and solubility testing.

Both in FaSSIF and FeSSIF, itraconazole concentrations increased with increasing CD concentrations, indicating that the solubility of itraconazole significantly benefits from inclusion of the compound in the CD cavity (Figure 1). Brewster et al. already reported a positive effect of HP-β-CD on the solubility of itraconazole in several aqueous buffer systems (Marcus E. Brewster et al., 2008). An important observation is that the itraconazole solubility

in FeSSIF was consistently lower than the solubility in FaSSIF, except for the condition where no CD was present. In the latter case, the higher abundance of mixed micelles in FeSSIF results in a superior solubility of itraconazole as compared to the simulated fluids of the fasted state. On the other hand, in presence of CDs, the relatively high concentrations of bile salts and phospholipids appeared to exclude a proportion of the itraconazole from the CD cavity, resulting in a lower itraconazole solubility. Furthermore, the ratio of the concentration measured in FeSSIF and in FaSSIF, depicted in Figure 1 demonstrates that the relative importance of this itraconazole exclusion phenomenon decreased with increasing CD concentration. At a concentration of 2% HP-β-CD, the itraconazole solubility in FeSSIF is only 27% of the solubility in FaSSIF, whereas at a CD concentration of 16% this ratio is 88%.

The simulated intestinal fluids used in this first solubility study contain sodium taurocholate as the sole type of bile salt. Recently, Riethorst et al. published a detailed characterization of human intestinal fluids in the fasted and fed state and confirmed that in addition to the taurine conjugates, the glycine conjugates are well represented bile salts in these media (Riethorst et al., 2016).

To compare the ability of the different bile salts to expel itraconazole from the CD cavity, the solubility of itraconazole in 4% HP-β-CD was evaluated in presence or absence of four of the most prominent bile salts present in human intestinal fluids (Figure 2). A lower solubility of itraconazole was measured in presence of all bile salts tested, indicating that both taurine and glycine conjugates have the potential to exclude itraconazole from the CD cavity. Sodium taurocholate (TC), the bile salt that is most commonly used in simulated intestinal fluids, appeared to cause the strongest drop in the solubilizing capacity of HP-β-CD. This is remarkable as the stability constants of the taurocholate - HP-β-CD complex reported by Schönbeck et al. were lower than the stability constants measured for glycodeoxycholate (GDC) and glycochenodeoxycholate (GCDC) (Schönbeck et al., 2010). Apparently, these higher

stability constants for some of the glycine conjugates, do not result in a stronger impact on the solubilizing capacity of HP-β-CD for itraconazole.

Although it can be useful to consider the impact of isolated intraluminal substances such as bile salts and phospholipids on the solubilizing capacity of a CD containing formulation, it is important to acknowledge the fact that mutual interactions between these substances may severely affect the outcome of the experiment. Indeed, aqueous media containing both bile salts and phospholipids are known to be highly different from simple bile salt solutions. Both size and solubilization capacity of bile salt micelles tend to increase significantly when phospholipids are added to the solvent system, generating mixed micellar media (Hörter and Dressman, 2001). To evaluate the effect of the presence of phosphatidylcholine on the solubilizing capacity of the CD containing biorelevant media, the solubility of itraconazole in aqueous buffers containing increasing concentrations of HP-β-CD was compared with its solubility under experimental conditions in which only taurocholate or both taurocholate and phosphatidylcholine were added to the CD containing medium (Figure 3). Interestingly, while the solubility of itraconazole in presence of both taurocholate and phosphatidylcholine was consistently lower than in the control situation (phosphate buffer, pH 6.5), this was not the case when only taurocholate was added to the buffer. Despite the fact that equal concentrations of taurocholate (10 mM) were present in the two micellar media, the addition of phosphatidylcholine clearly induced a shift in the equilibrium interplay between itraconazole, HP-β-CD and the micellar fraction in the solvent system: at the lower CD concentrations of 1 and 2%, itraconazole solubility in presence of both taurocholate and phosphatidylcholine is significantly lower than in presence of only taurocholate. This empirically observed divergence between mixed micellar media and media containing only bile salt micelles, advocates a cautious interpretation of in vitro or mathematical models that do not fully appreciate the complex nature of biorelevant media.

When the CD concentration increases further up to 4%, the impact of the additional presence of phosphatidylcholine appears to abate. This is in accordance with the findings presented in Figure 1, where the impact of the concentration of biorelevant components decreased with increasing CD concentrations.

3.2 Displacement of itraconazole from HP-β-CD: supersaturation/precipitation behavior

Solubility studies are useful as they offer valuable information about the itraconazole concentrations that can be expected when the system is in equilibrium. In the dynamic environment of the gastrointestinal tract, however, a formulation is subjected to multiple processes and drugs are often in a thermodynamically unstable state. Supersaturation in human intestinal fluids has been described for several compounds, both in vivo and in vitro (Bevernage et al., 2013; Psachoulias et al., 2011; Stappaerts et al., 2015).

In an effort to evaluate the immediate effects of bile secretion on the intestinal behavior of itraconazole in presence of HP-β-CD, a displacement study was performed in which a concentrated solution of taurocholate and phosphatidylcholine was titrated into a solution containing 20 µM of itraconazole in 4% HP-β-CD. Figure 4 depicts the concentrations that were measured upon adding increasing amounts of taurocholate and phosphatidylcholine. Notwithstanding the fact that with increasing concentrations of bile salts and phospholipids the solubilizing capacity of the medium, determined by solubility studies as described in the previous section, dropped significantly (triangles in Figure 4), itraconazole remained in solution (squares in Figure 4). Even 100 min after the addition of the highest amount of taurocholate and phosphatidylcholine tested, no precipitation of itraconazole could be demonstrated. This is interesting, as these findings support the mechanism of itraconazole supersaturation induction through secretion of bile and subsequent competition between itraconazole and bile constituents for the CD cavity.

A similar displacement study was performed to evaluate the short term effects of the presence of different bile salts on the behavior of itraconazole. Analogously to the concentrated simulated intestinal fluid solution used in the previous experiment, a concentrated bile salt solution was titrated into a 4% HP-β-CD solution containing 40 µM of itraconazole. The targeted bile salt concentration upon addition of the concentrated solution was 10 mM, which is situated between the bile salt concentrations used in FaSSIF and FeSSIF and is well within ranges reported in man (Riethorst et al., 2016).

Figure 5 displays the itraconazole concentrations measured in the CD containing media after addition of the different bile salts. Based on the solubility of itraconazole in these media, precipitation of itraconazole would be expected (Figure 2). For three of the four bile salts tested (the glycine conjugates), no precipitation was observed over a period of 120 min. Only for taurocholate, minor precipitation was observed, clearly indicating that competition between itraconazole and the bile salts is genuinely taking place in the medium causing itraconazole to be partially displaced from the CD cavity. A comparison with the solubility experiment illustrated in Figure 2 shows that in presence of bile salts, concentrations of itraconazole in the displacement study consistently exceed the apparent solubility values measured after 24 h, again confirming generation of itraconazole supersaturation upon displacement from the CD cavity.

A supersaturated solution is metastable and under the experimental conditions described here, itraconazole will eventually precipitate. In vivo, however, the supersaturated fraction of itraconazole, being excluded from the CD cavity, is freely bioaccessible and may be immediately absorbed by the enormous surface area of the small intestine. Therefore, the combination of solubility and displacement studies is a useful approach to predict the performance of CD containing formulations in a biorelevant context.

3.3 Gastrointestinal transfer studies

The impact of the stomach on the intestinal absorption of both basic and acidic compounds has been abundantly described (Brouwers et al., 2007; Van Den Abeele et al., 2016; Walravens et al., 2011). As compared to their solubility in intestinal fluids, basic compounds generally exhibit a superior solubility in the low pH ranges of the gastric fluids. Recent clinical findings from our research group clearly demonstrate gastrointestinal transfer to result in itraconazole supersaturation in the small intestine (manuscript in preparation). To include this dynamic process in our mechanistic study, the behavior of itraconazole following gastrointestinal transfer was studied by performing in vitro transfer experiments in presence of relevant concentrations of HP-β-CD and varying concentrations of bile salts and phospholipids. The gastric compartment contained increasing concentrations of HP-β-CD saturated with itraconazole, the highest of which amounted to 12%. This percentage was calculated based on the intake of 20 ml of a 40% HP-β-CD containing itraconazole solution (cfr. Sporanox®) and a residual gastric volume of 50 ml. The in vivo transfer from the stomach to the small intestine was simulated by a 1:1 dilution from the compartment containing the simulated gastric fluids into the intestinal compartment containing a double concentrated phosphate buffer in presence or absence of varying concentrations of taurocholate and phosphatidylcholine. The dilution factor of 1:1 was selected in accordance with a study reported by Hens et al. describing the gastrointestinal transfer of a solution containing the non-absorbable marker paromomycin (Hens et al., 2014). In the intestinal compartment, different concentrations of taurocholate and phosphatidylcholine were used. The selection of taurocholate and phosphatidylcholine seemed reasonable based on the observed discrepancy in solubilizing capacity of HP-β-CD in presence of bile salts alone or in media containing both taurocholate and phosphatidylcholine (Figure 3), combined with the observation that taurocholate displayed the strongest impact on itraconazole displacement (Figure 2 and Figure 5).

In a first step, the solubility of itraconazole was determined in simulated gastric fluids and in the blank transfer media. These are the media resulting from the 1:1 dilution of SGF containing the selected concentrations of HP-β-CD in double concentrated phosphate buffer (pH 7.5) supplemented with the selected concentrations of bile salts and phosphatidylcholine (Table 1). Solubility values in presence of equal CD concentrations were significantly higher in the simulated gastric fluids than in the simulated intestinal fluids. Brewster et al. suggested that at low pH values, both drug protonation and changes in the interaction between itraconazole and HP-β-CD are responsible for the high solubility of itraconazole in gastric media containing HP-β-CD (Marcus E. Brewster et al., 2008). In all CD containing intestinal media tested, itraconazole solubility decreased with increasing concentrations of bile salts and phospholipids, again confirming the exclusion of itraconazole from the CD cavity by the constituents of the biorelevant fluids. Evidently, in intestinal media containing equal amounts of phospholipids and bile salts, solubility increased with increasing CD concentration.

Table 1: Apparent solubility of itraconazole in simulated gastric fluids and simulated intestinal fluids (blank transfer medium) in presence of increasing concentrations of HP-β-CD. Simulated intestinal fluids with various concentrations of taurocholate and phosphatidylcholine were tested. Solubility values are presented in µM and represent mean ± SD (n=3).

SGF		SIF	0 mg/ml	2.5 mg/ml	7.5 mg/ml	10 mg/ml
0% HP-β-CD	7.4±1.3	**0% HP-β-CD**	4.5±2.5	1.3±0.2	1.0±0.1	1.2±0.1
2% HP-β-CD	84.2±5.2	**1% HP-β-CD**	4.2±1.0	2.3±0.3	1.8±0.2	1.6±0.3
4% HP-β-CD	233.1±18.5	**2% HP-β-CD**	10.4±0.3	5.2±0.3	2.9±0.2	2.7±0.1
8% HP-β-CD	627.7±21.8	**4% HP-β-CD**	28.6±5	16.4±0.4	9.6±0.5	8.0±0.6
12% HP-β-CD	1051.5±68.9	**6% HP-β-CD**	47.3±5.3	32.4±0.6	21.9±0.8	19.3±0.4

Figure 6 presents an overview of the transfer experiments that were conducted: solubility values in the transfer medium are depicted as well as the concentrations that were measured in the intestinal compartment in function of time. For all conditions, a similar pattern could be observed in the concentration-time profiles: a peak concentration was measured in the earliest time point after which the itraconazole concentrations dropped steadily, pursuing the apparent solubility. Regardless of the concentrations of HP-β-CD, taurocholate and

phosphatidylcholine present in the intestinal compartment, a period of itraconazole supersaturation could be demonstrated. The duration of this period as well as the extent of supersaturation increased strongly with increasing concentration of HP-β-CD. This observation can be explained by the ability of hydrophilic CDs to function as a precipitation inhibitor maintaining supersaturation (M. E. Brewster et al., 2008).

Interestingly, a concentration dependent impact of bile salts and phospholipids on the itraconazole concentration-time profiles was demonstrated (grey versus black bars in Figure 6). While itraconazole concentrations in presence of taurocholate and phosphatidylcholine at the earliest time point were at least equally high as the concentrations in absence of these bile constituents, solubilized concentrations of itraconazole decreased at a higher rate in the condition with the highest concentrations of bile salts and phospholipids tested (10 mg SIF/ml) than in the control condition (0 mg SIF/ml). At moderate concentrations (2.5 mg SIF/ml), the concentration of itraconazole was consistently similar to the control condition. These findings indicate that high concentrations of bile salts and phospholipids may drive itraconazole precipitation as they are able to expel itraconazole from the CD cavity.

In addition to the absolute concentrations of itraconazole, Figure 6 depicts the degrees of supersaturation (i.e. the itraconazole concentrations measured after the transfer step divided by their apparent solubility) that are reached in the intestinal media. Especially at the early time points and for the highest concentrations of bile constituents, high degrees of supersaturation are reached. The lower apparent solubility as compared to the control condition combined with the similar concentrations measured at the earliest time points, results in degrees of supersaturation as high as 12, 14 and 12 for the condition with the highest taurocholate and phospholipid concentration in presence of 2, 4 and 6% of HP-β-CD, respectively. These degrees of supersaturation are much higher than in absence of bile constituents. Moreover, the extent of supersaturation (i.e. the AUC of the concentrations measured over the period of 100 min minus

the AUC of the apparent solubility maintained over 100 min) in presence of bile constituents also supersedes the extent of supersaturation in absence of bile salts and phospholipids. As the supersaturated fraction of itraconazole is readily bioaccessible and escapes the solubility-permeability trade-off, the effect of endogenous compounds on supersaturation/precipitation behavior of a drug in presence of CDs is very likely to affect the absorption rate and the extent of absorption of drugs.

4 Conclusion

The results of this study demonstrate the importance of evaluating formulation performance in media that reflect an environment that is relevant for the site where the formulation has its impact on bioavailability. Data clearly indicate that competing agents present in the small intestine may diminish the solubilizing capacity of CDs. Bile salts and phospholipids were shown to exclude itraconazole from the CD cavity of HP-β-CD in solubility studies. Moreover, displacement studies and gastrointestinal transfer studies, designed to reflect the dynamic nature of the gastrointestinal environment, revealed the ability of these competing agents to displace solubilized itraconazole from the CD cavity. Comparison with solubility data learned that this displacement can trigger supersaturation possibly leading to improved intestinal absorption.

Acknowledgement

This work has received support from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT) (Grant No. 135040).

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Legend for figures

Figure 1: Apparent solubility of itraconazole in FaSSIF (white bars) and FeSSIF (black bars). Open diamonds represent the ratio of the solubility of itraconazole in FaSSIF and in FeSSIF. Bars represent the mean + SD (n=3)

Figure 2: Apparent solubility of itraconazole in 4% of HP-β-CD in presence or absence of 10 mM of TC, GC, GDC or GCDC. Bars represent mean + SD (n=3).

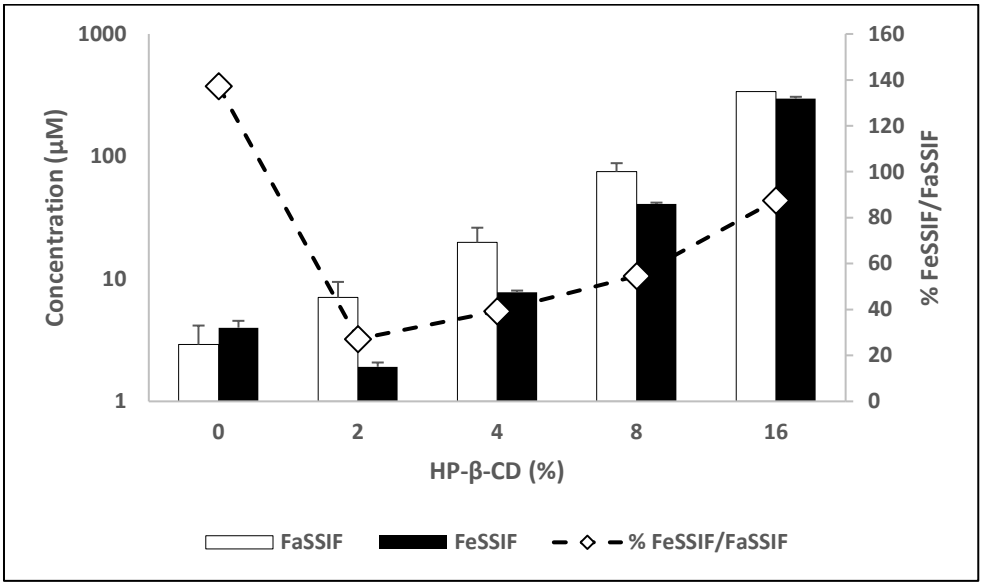
Figure 3: Apparent solubility of itraconazole in phosphate buffer (white bars), phosphate buffer + 10 mM taurocholate (grey bars) or phosphate buffer + 10mM taurocholate and 2.5 mM phosphatidylcholine in presence of increasing concentrations of HP-β-CD. Bars represent mean + SD.

Figure 4: Triangles represent the apparent solubility of itraconazole in 4% of HP-β-CD in a phosphate buffer in presence of increasing concentrations of taurocholate and phosphatidylcholine. Squares represent the concentrations measured in the displacement study, 100 min after the addition of concentrated solutions of SIF powder to a phosphate buffer containing 20 µM of itraconazole in a 4% HP-β-CD solution in a phosphate buffer. Symbols represent mean ± SD (n=3).

Figure 5: Concentrations measured during the displacement study in function of time upon the addition of concentrated bile salt solutions to a phosphate buffer containing 40 µM of itraconazole in a 4% HP-β-CD solution in a phosphate buffer. Bars represent mean ± SD (n=3).

Figure 6: Apparent solubility measured in blank transfer medium and time dependent concentrations measured in the intestinal compartment during the gastrointestinal transfer studies in absence of SIF powder (white bars), in presence of 2.5 mg SIF powder per ml of medium (grey bars) and in presence of 10 mg SIF powder per ml of medium (black bars). The degrees of supersaturation (DS) for itraconazole in absence (squares) and in presence of 2.5 (crosses) and 10 (circles) mg SIF powder per ml of medium are depicted as well. Bars represent mean + SD (n=3).

542 Figure 1



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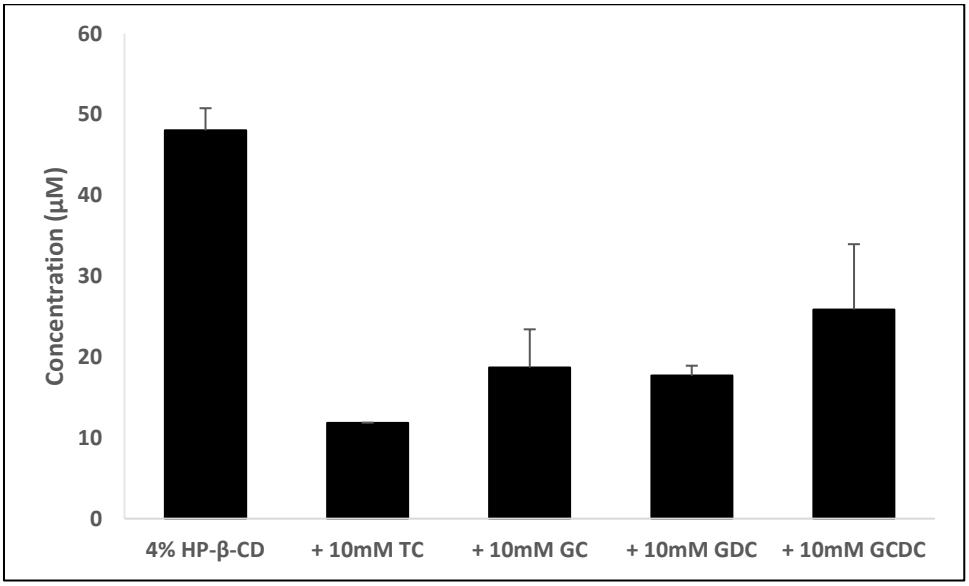
544 Figure 2



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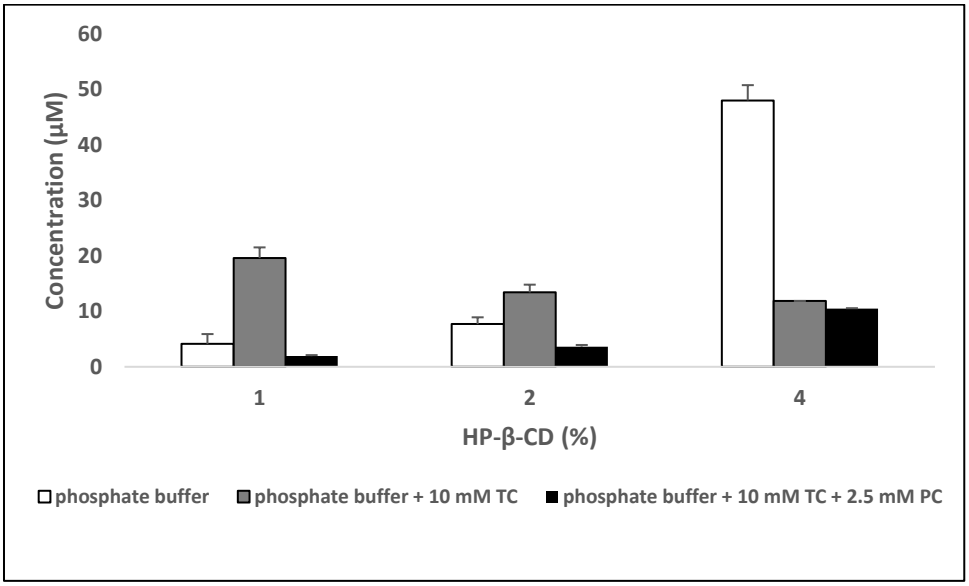
547 Figure 3



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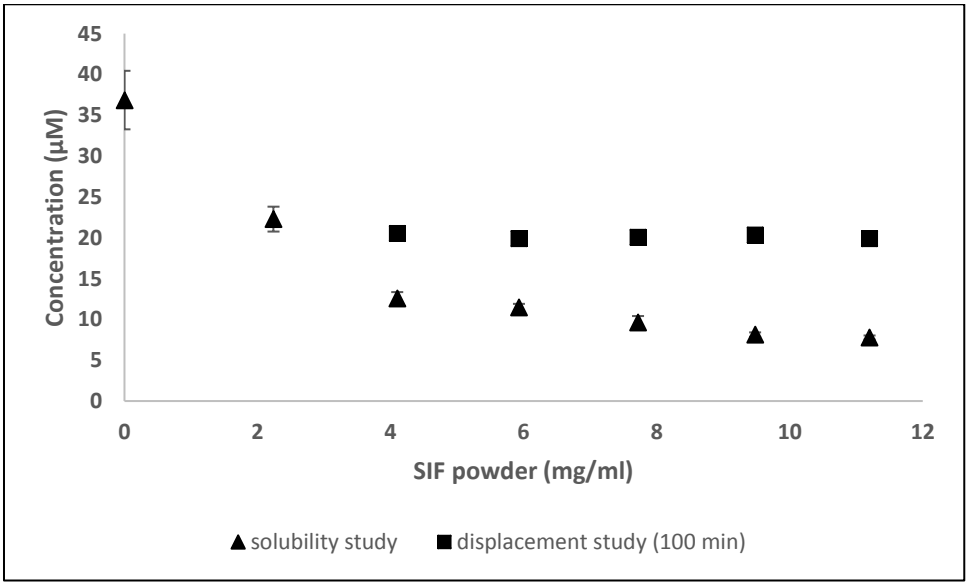

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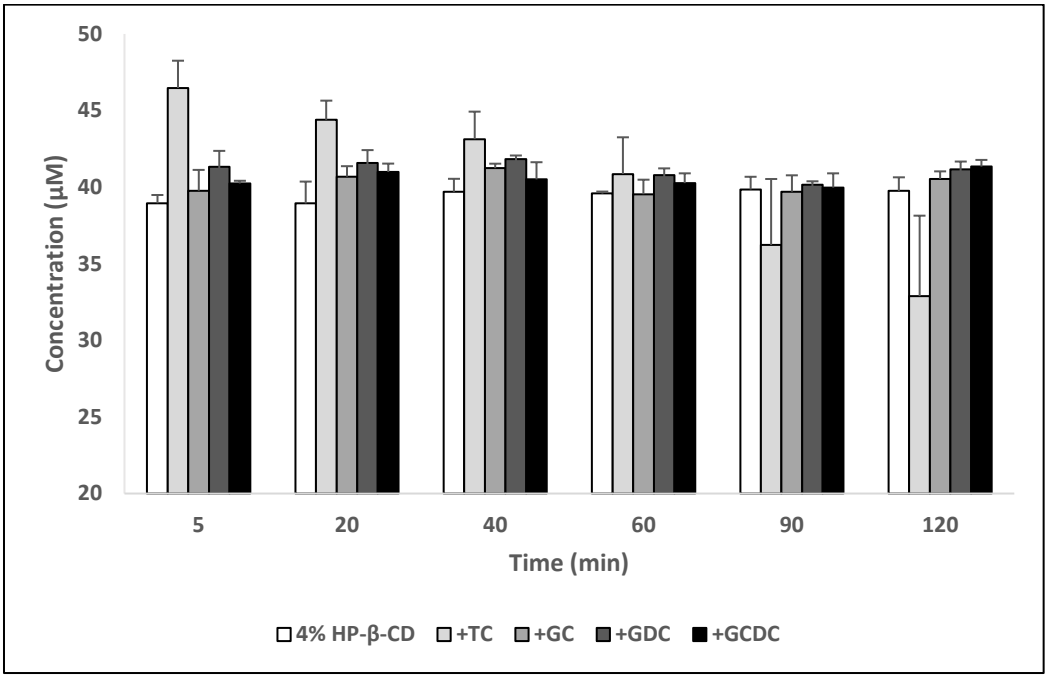
552 Figure 5



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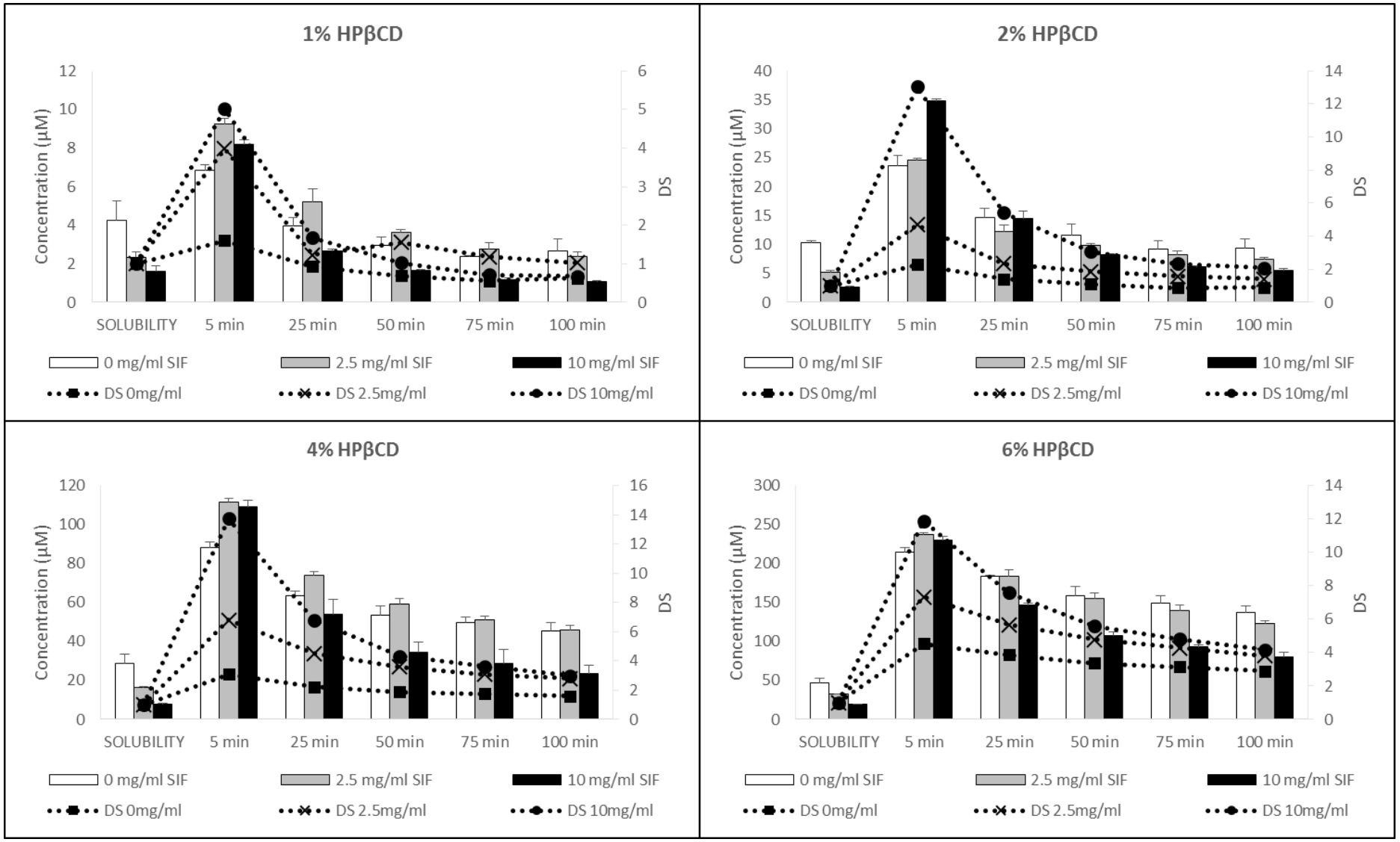
555 Figure 6



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